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Peripheral neuro-immune pathology in recessive dystrophic epidermolysis bullosa

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To the editor

Chronic pain and itch are substantial quality-of-life obstacles for patients with the genetic skin disorder recessive dystrophic epidermolysis bullosa (RDEB). RDEB is caused by loss-of-function mutations in the anchoring fibril protein type VII collagen. Extreme skin fragility leads to chronic wounds and inflammation that is accompanied by significant pain and itch. Itchy skin has consistently rated as the highest burden in RDEB patients (Danial *et al.*, 2014; van Scheppingen *et al.*, 2008), and another study found that 93% of dystrophic EB patients experienced itch symptoms (Snauwaert *et al.*, 2014). Compounding the burden of itching in RDEB, scratching can be associated with new lesions and secondary infection, exacerbating the disease's symptoms and undermining treatment.

The second-highest burden in RDEB is pain (Danial *et al.*, 2014; van Scheppingen *et al.*, 2008). Fifty percent of RDEB patients rated their daily pain level as at least a 5 on a scale of 0–10, and only 5% of patients were without pain (Goldschneider and Lucky, 2010). Pain symptoms in RDEB are often extreme and resistant to first-line analgesics. More potent opioid analgesics are effective against moderate to severe pain, but can exacerbate itch (Goldschneider and Lucky, 2010), making them potentially unbearable or unacceptable treatments. Here, we describe distinct peripheral nerve morphology and mast cell accumulation in RDEB skin. These findings are an important step toward understanding the mechanisms of pain and itch in dystrophic EB, which should point the way toward more effective analgesics and antipruritics.

We first characterized the epidermal nerve fiber (ENF) density, a widely used metric for peripheral neuropathy, in six RDEB patients in comparison with six healthy control subjects

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(all samples were obtained with written, informed patient consent). All RDEB patients rated itch and pain as at least a 4 (“very bothersome”) on the 5-point Likert scale (Danial *et al.*, 2014), with an average itch rating of 4.5 ± 0.55 and an average pain rating of 4 ± 0 (Supplementary Tables I–II). Skin biopsies taken from perilesional sites on individuals with RDEB or from healthy controls were stained with antibodies against protein gene product 9.5 (PGP9.5), a pan-neuronal marker commonly used to identify ENFs (Chiang *et al.*, 2011; Kennedy *et al.*, 1996). PGP9.5-positive nerves within the epidermis were traced in 3-dimensional confocal images (Kennedy *et al.*, 1996).

RDEB patients had significantly fewer ENFs than healthy control subjects. Epidermal innervation decreases with age (Panoutsopoulou *et al.*, 2014), thus ENF density for each patient was plotted against his or her age. Since epidermal separation during tissue processing is common in RDEB skin, we confirmed that age-dependent ENF densities were reduced in RDEB skin both when considering only unblistered skin regions (Figure 1a) as well as with all regions included (Figure 1b). All RDEB patients also had marked dermal nerve disorganization. Two patients lacked more than 75% of nerve density in the subepidermal neural plexus (SNP) (Figure 1c) and all patients lacked normal SNP morphology (Figure 1d) and bundled nerve structures in the deep dermis fibers (Supplementary Figure S1). Total PGP9.5 immunofluorescence in the dermis of RDEB patients was significantly reduced over control skin both as a group (Figure 1e) and as a function of age (Supplementary Figure S2).

The critical next step is to understand how these morphological peripheral nervous system (PNS) changes arise and impact a patient’s sensory experience. In atopic dermatitis (AD), lesional keratinocytes make more nerve growth factor and less of the axonal repellent semaphorin 3A, causing greater ENF densities and itch sensitivity (Tominaga and Takamori, 2014). While other pruritic and painful conditions have subnormal numbers of ENFs (Caro and Winter, 2014; Kennedy *et al.*, 1996; Maddison *et al.*, 2011; Schuhknecht *et al.*, 2011), the mechanisms are less well understood. Extended periods of inactivity and systemic opioid analgesia in RDEB may be factors. Abnormal innervation could be associated with fibrotic changes present in RDEB skin. Itching is common in fibrotic scar tissue, and, while increased innervation has been described in hypertrophic scars and animal models (Liang *et al.*, 2004; Zhang and Laato, 2001), another study found fewer epidermal nerve fibers in itchy keloids (Tey *et al.*, 2012).

Alternatively, the epidermal hypoinnervation in RDEB, in combination with the lack of visible scarring at the biopsy sites, might be indicative of an intrinsic axonal guidance dysfunction. In support of this hypothesis, proteomic analysis of RDEB extracellular matrix (ECM) has revealed decreased matrix metalloproteinase-2 activity (Kuttner *et al.*, 2013), which is involved in neuronal growth cone formation (Tominaga *et al.*, 2009), and decreased basement membrane proteins, including several laminin chains. Laminin-332, the protein absent in the junctional form of EB (JEB), has been shown to inhibit nerve branching as well as neuronal graded potentials *in vitro*, which is consistent with an increase in ENFs in JEB patients (Chiang *et al.*, 2011). Therefore, perturbations in ECM homeostasis caused by loss of type VII collagen may dictate sensory pathology in a systemic, developmental fashion rather than through secondary skin injury.

Cutaneous mast cells (MCs) are significant sources of neurotrophic factors such as NGF, and MC-derived tumor necrosis factor- α is required for epidermal hyperinnervation in a rodent model of atopic dermatitis (Kakurai *et al.*, 2006). MC degranulation alone can trigger pain responses (Chatterjea *et al.*, 2012; Drummond, 2004) and MCs are the most significant source of histamine and other pruritic compounds such as tryptase. To consider their role in RDEB, we quantified MCs in patient biopsies using tryptase immunostaining. Four out of six RDEB biopsies contained significantly more MCs, especially in the upper dermis (Figure 2a–b), and half also contained higher amounts of free MC granules (Figure 2c), which were more localized to the lower dermis (Figure 2a), in comparison to control skin. Given these findings, an MC stabilizer such as sodium cromolyn (sodium cromoglycate) may be effective for treatment of pain or itch in some patients.

In summary, all RDEB patients in this study had significant peripheral nerve pathology, and 67% (4/6) had increased dermal MC numbers and degranulation. To our knowledge both the pattern of PNS pathology and MC activity in RDEB have not been reported previously, and can serve as a platform for rational identification of effective analgesic and antipruritic treatments in a disease for which they are desperately needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

RDEB	recessive dystrophic epidermolysis bullosa
ENF	epidermal nerve fiber
SNP	subepidermal neural plexus
PGP9.5	protein gene product 9.5
MC	mast cell
JEB	junctional epidermolysis bullosa
PNS	peripheral nervous system
NGF	nerve growth factor

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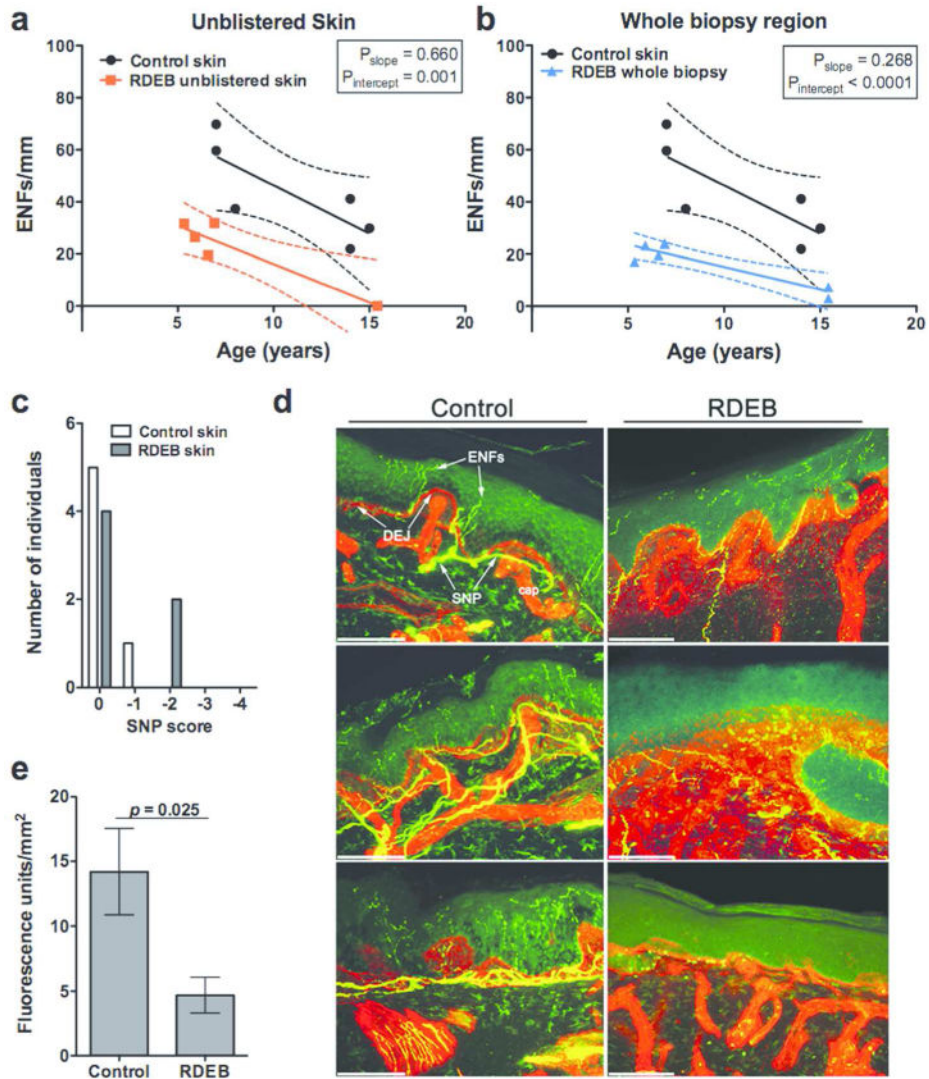


Figure 1. RDEB patients have fewer epidermal nerve fibers and disorganized dermal nerve architecture

(a–b) To assess epidermal nerve fiber (ENF) density, ENFs were manually traced in 32 μ m confocal image stacks of formalin-fixed skin sections stained with anti-PGP9.5 rabbit antiserum and anti-collagen IV goat IgG. Slopes and intercepts within the 95% confidence interval of the linear regression were compared between groups to determine significance. Dotted lines indicate 95% confidence interval of the linear regression fit. (c) PGP9.5-stained skin sections were scored for nerve content in subepidermal neural plexus (SNP score), where 0 = normal, $-1 = \frac{1}{2}$ normal levels, $-2 = \frac{1}{4}$ normal levels, $-3 = < \frac{1}{4}$ normal levels, and 4 = no nerve. (d) 20 \times images of PGP9.5-positive nerves in the upper dermis and epidermis (scale bar = 50 μ m, arrows indicate SNP-associated nerves). (e) As another measure of the subepidermal neural plexus region, total units of PGP9.5 immunofluorescence per square millimeter was quantified within the first 200 μ m of dermis below the dermal-epidermal junction, plotted as mean \pm SEM ($n = 6$), and groups compared by t-test. Supplementary materials contain detailed methods. DEJ = dermal-epidermal junction; SNP = subepidermal neural plexus; ENFs = epidermal nerve fibers; cap = capillary

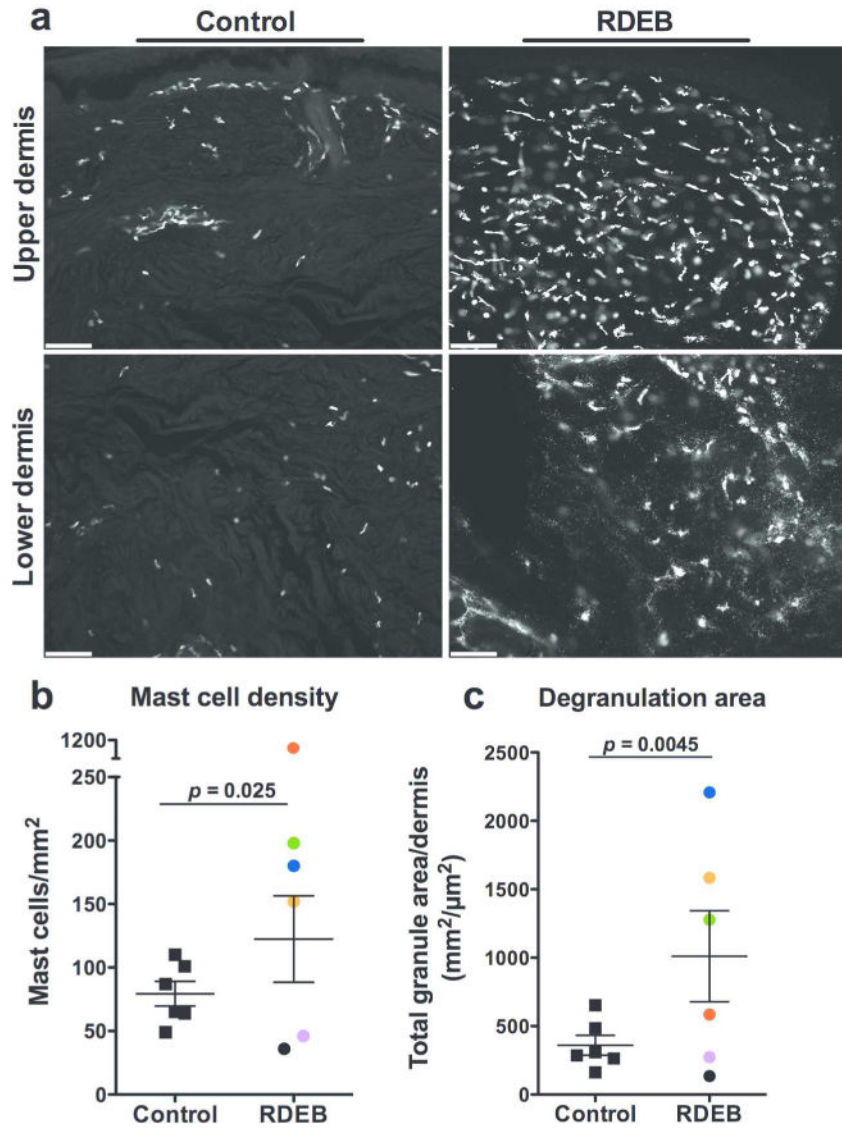


Figure 2. Increased mast cell number and degranulation in RDEB patients
 (a) Mast cells in RDEB and control skin were visualized by immunostaining with mouse anti-tryptase antiserum (Millipore) in 60µm sections (scale bar = 100µm). (b) Mast cell density, and (c) degranulation area were quantified in NeuroLucida through automated object tracing, and groups compared by t-test. Error bars display the mean +/- SEM. Supplementary materials contain detailed methods. Data from patients are color-coded in the same fashion for graphs b–c. One patient (shown in red) had substantially more mast cells than any other individual and, to assess the data in the most conservative fashion, has not been included in the t-test result shown (were the data point included $p < 0.001$).